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LYSIS OF ESCHERICHIA COLI BY MARINE MICROORGANISMS

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Harvard University
Cambridge, Massachusetts

April 1968

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Lysis of Escherichia coli by Marine Microorganisms

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Estuarine pollution has increased dramatically in recent years with increased urbanization. The pollution of our coastal regions with domestic sewage frequently leads to contamination of shellfish beds and subsequent outbreaks of hepatitis. In addition, the danger to bathers is often extensive.

When the outflow of sewage is small enough so that the sewage microorganisms do not completely dominate the marine microflora, those microorganisms carried in sewage are rapidly killed in the sea. A number of different hypotheses have been proposed to account for this kill including dilution, heavy metals, unknown organic compounds, and algal products. The object of this research was to determine precisely the mechanism of kill of intestinal microorganisms in the sea. Most of the data obtained have been published or are in the process of publication. Reprints and preprints are included in this report. The information in these publications is summarized below:

1. It was observed that the rate and extend of kill of Escherichia coli in natural seawater is proportional to the size of the native microbial population in the water, i.e. the marine microflora is directly responsible for the kill of intestinal bacteria in the sea.

2. Repeated inoculations of E. coli into the same sample of seawater causes a shortening of the lag phase for the kill and increases the rate of kill. These data indicate that in seawater regularly exposed to sewage the rate of kill is more rapid than in water irregularly exposed to sewage. However, the data summarized in (1) indicate that in heavily polluted water where the native microflora is superceded by the sewage microflora, the self-purification process is completely inhibited.

3. Filtrates (0.45μ) of natural seawater displayed increased activity against E. coli with each subsequent exposure to that bacterium. Analyses

of the filtrates showed an enrichment of a filterable bacterium, Bdellovibrio bacteriovorus, which is parasitic on E. coli. This parasitic organisms is very tiny, appears to be quite common in marine estuaries and rapidly attacks E. coli and other intestinal bacteria. It does not grow on the nutrient media usually used in the laboratory.

A survey of the marine microorganisms capable of killing E. coli showed that bacteria which enzymatically degraded E. coli cell walls are common in the sea. However, a more detailed investigation disclosed that these bacteria are similar to the antibiotic producers, also common in the sea, in that under natural conditions they do not actively kill intestinal bacteria.

Recent studies in our laboratory have shown that another obligately parasitic microorganism is involved in the killing of intestinal bacteria in the sea. This is a very small marine amoeba, as yet unclassified. It develops rapidly in all samples of seawater to which E. coli is added and is capable of killing large numbers of intestinal bacteria.

4. Pathogenic fungi are frequently carried into the sea in sewage. Our study included an investigation of the mechanism of kill of fungi in the sea. This appeared to be much more simple than the killing process for intestinal bacteria. Addition of non-marine fungi to seawater resulted in a stimulation of marine bacteria which lysed their cell walls, thus killing the fungi. No other processes were detected.

5. We have recently initiated studies to determine the significance of flocculation in the killing of E. coli in the sea. Results are not yet available for this phase of the study. The function of E. coli cell walls and of polymers produced by marine microorganisms in the flocculation process are under investigation.

6. Our recent publications included in this report have stimulated renewed interest in the problems involved in self-purification in the sea. Dr. Mitchell has recently been asked to review the topic "Factors affecting the decline of non-marine microorganisms in the sea for the Journal of the International Water Pollution Research Association, Water Research. Part of this review is being written during a semester's leave currently being spent at the Scripps Institution of Oceanography. During this stay in California, seminars have been given on this topic at the Scripps Institution, at Stanford University, the University of California at Berkeley, and California Institute of Technology. The increasing problems of ocean disposal of sewage off California has stimulated a great deal of interest in this subject and these seminars have led to a much closer association between our more fundamental studies and field studies being carried out by engineers.

An interest in the self-purification of the ocean is also being renewed among microbiologists. Dr. Mitchell has spent two summers working as a guest in Dr. Jannasch's laboratory at the Woods Hole Oceanographic Institution. Jannasch is an international authority on the continuous culture of microorganisms. A paper was published together with Jannasch entitled "Lysis of Escherichia coli by Marine Mocroorganisms" in the English scientific journal "Nature". Together with Jannasch and other scientists, Dr. Mitchell has been invited to take part in a round table discussion entitled "Processes affecting the Decline of Intestinal Bacteria in the Sea" at the 1968 annual meeting of the American Society for Microbiology to be held in May. This forum reflects renewed interest in this problem by microbiologists. Little or no research has been carried out in this area by microbiologists for at least the past five years.

7. The practical implications of this research for the control of pollution in the sea have yet to be developed. Discussions with engineers have indicated that knowledge of the processes causing the killing of intestinal microorganisms in the sea immeasurably helps in the design of sewage outfalls.

Another interesting aspect of this work yet to be developed is the analogy to fresh water. Preliminary data from our laboratory indicate that similar mechanisms are operative in the self-purification of fresh water. We intend to investigate this aspect during the next year.

List of Publications

1. Mitchell, R., Yankofsky, Shlomit, and Jannasch, H.W., "Lysis of Escherichia coli by Marine Microorganisms" Nature, 215: 891 (1967).

2. Mitchell, R. and Wirsen, C., "Lysis of Non-marine Fungi by Marine Microorganisms." J. Gen. Microbiol. In Press (1968).

3. Wirsen, C. and Mitchell, R., Bacterial Lysis of Non-marine Microorganisms in Seawater." Proceedings of the Conference on Drugs from the Sea, 1968. Tetrahedron Letters (In Press).

4. Mitchell, R., "Lysis of E. coli by Marine Microorganisms" Bacteriol. Proc. G50 (1967). (Paper presented at the American Society for Microbiology Meeting, New York, 1967).

5. Mitchell, R., The Effect of Water Movement on Lysis of Non-marine Microorganisms by Marine bacteria. Sarsia In Press 1968. (Paper present at the 2nd European Symposium on Marine Biology, Bergen, 1967).

6. Mitchell, R. and Morris, J.C., The Fate of Intestinal Bacteria in the Sea. (Paper to be presented at the International Water Pollution Research Conference, Prague, 1968).

7. Mitchell, R., Lysis of Escherichia coli by Marine Microorganisms, Internl. J. Water Research In Press (1968). (A review Requested by the editors of Water Research).

(*Reprinted from Nature, Vol. 215, No. 5103, pp. 891–893, August 19, 1967*)

Lysis of *Escherichia coli* by Marine Micro-organisms

INVESTIGATIONS of the lethal effect of seawater on *Escherichia coli*, the common indicator organism for pollution by intestinal pathogens, have suggested a variety of possible mechanisms[1–3]. The negative effect of heat sterilization of seawater on its specific bactericidal action indicates a biological interaction which has been neither confirmed nor disproved. We attempt to define here the role of the native microflora in the disappearance of *E. coli* from seawater.

We studied the relationship between the size of the marine microbial population and the rate of death of *E. coli* using seawater concentrates of different population densities. Fresh seawater, sampled at Nahant and Woods

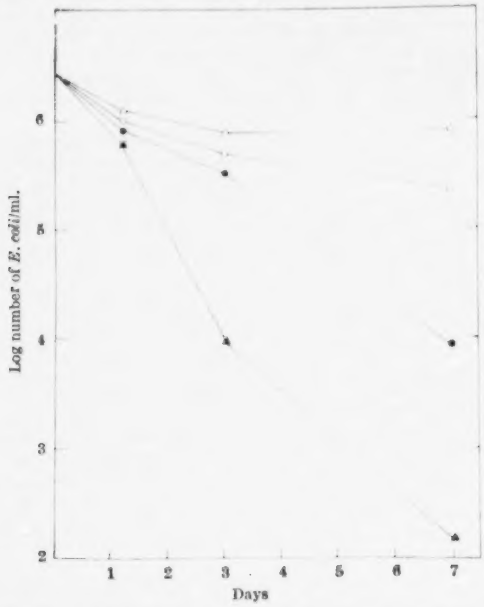

Fig. 1. Plot of the log of the number of *Escherichia coli* present in autoclaved seawater after the addition of different concentrations of marine micro-organisms. △, Autoclaved seawater; ○, autoclaved seawater plus ten marine bacteria/ml.; ●, autoclaved seawater plus 10^3 marine bacteria/ml.; ▲, autoclaved seawater plus 10^5 marine bacteria/ml.

Hole, Massachusetts, was centrifuged at 4,000*g* at 10° C and the bacterial count of a series of concentrates was determined by surface plating on a peptone–yeast extract agar[4]. 100 ml. quantities of autoclaved seawater to which different concentrations of marine micro-organisms had been added were placed in 250 ml. Erlenmeyer flasks. Strain K12 of *E. coli* was grown in nutrient broth and was inoculated into all flasks at a concentration of 10^6/ml. The mixture was incubated at 26° C on a rotary shaker. Viable counts of *E. coli* were made at regular intervals on eosin methylene blue agar at 37° C.

The results (Fig. 1) show that the decrease in numbers of *E. coli* was strongly affected by the size of the marine microbial population. Almost no decrease was detected in autoclaved water. As the concentration of marine bacteria in the water increased, so also did the rate and extent of death of *E. coli*.

In parallel experiments we investigated the survival of *E. coli* in samples of seawater taken from the Atlantic Ocean 150 miles off Cape Cod, Massachusetts. Populations of *E. coli* added to the water declined from 10^9 cells/ml. to 10^7 cells/ml. in 5 days when the total marine bacterial population was about 10^3 bacteria/ml. By comparison, the population of *E. coli* declined from 10^9/ml. to 10^4/ml. in 5 days in seawater sampled from the harbour at Woods Hole, Massachusetts, which had a bacterial population of about 10^6/ml.

The development of a specific lytic microbial population after inoculation of *E. coli* into seawater was indicated by the results of later experiments, in which *E. coli* was re-inoculated to natural seawater 5 days after the initial inoculum. As shown in Fig. 2, the lag phase of the death curve of *E. coli* was virtually eliminated in the second curve. The number of *E. coli* cells declined from 10^6/ml. to 10^2/ml. in 4 days following the second inoculation, compared with 7 days following the initial inoculation into seawater. The indigenous marine bacterial population remained constant at 10^4/ml. throughout the experiment.

The stimulation of the lethal activity of seawater by the addition of *E. coli* suggested the enrichment of a microflora parasitic or lytic to *E. coli*. In later experiments, *E. coli* was added to natural seawater in Erlenmeyer flasks as before. Samples of the water were taken at daily intervals and placed on double layer plates containing autoclaved seawater and 1·5 per cent agar in the bottom layer. The top layer contained autoclaved seawater with 0·7 per cent agar and inoculated with 10^{10} cells of *E. coli* K12/ml.

Two kinds of responses were recorded after 2–3 days: (1) the appearance of bacterial colonies surrounded by a clearing zone in the bacterial lawn; (2) the appearance of clearing zones without visible colonies (plaques). Tests showed that the organisms isolated from the colonies were capable of utilizing the cell walls of *E. coli* as a sole source of organic carbon. Lysis was caused by degrada-

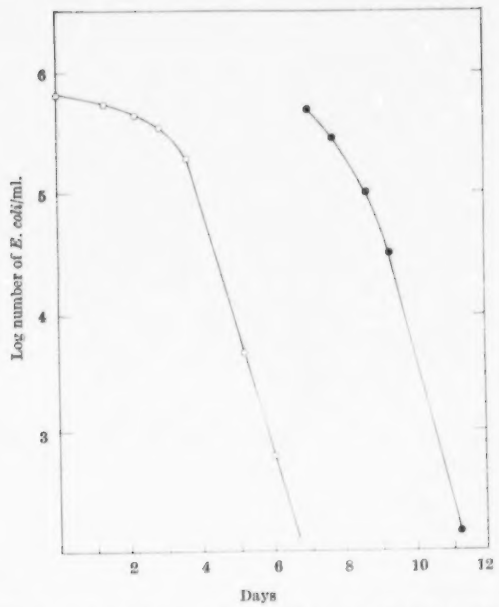

Fig. 2. Accelerated decline of the population of *Escherichia coli* in natural seawater with a re-inoculation of *E. coli* after 7 days. ○, First inoculum of *E. coli*; ●, second inoculum of *E. coli*.

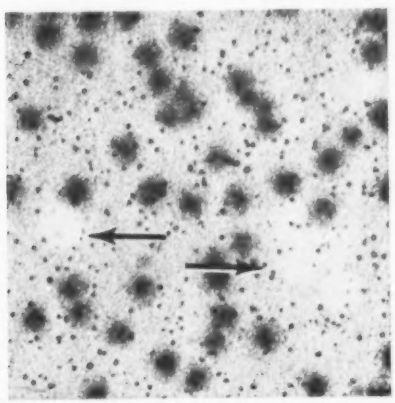

Fig. 3. Plaques observed when seawater exposed to *Escherichia coli* was inoculated onto double layer plates of autoclaved seawater and *E. coli*.

tion of the cell walls by extracellular enzymes. In a previous report[5] we described the isolation of similar bacteria from seawater and their activity in killing *E. coli* in artificial seawater.

After exposure of seawater to *E. coli* for 5 days, the number of plaques increased from about 10/ml. in natural water to 10^3/ml.—as measured by the double layer technique. In samples from the plaques, phase contrast microscopy revealed the presence of large numbers of highly motile vibrios ranging in size from 0·2 to 0·5μ. These bacteria did not grow on the peptone–yeast agar used in this study and seemed to be obligate parasites. They were also halophilic. Plaques regularly appeared in 3 days on *E. coli* K12 or B double layer plates at 22° C. Very few of these plaques were obtained with natural seawater, unless previously inoculated with *E. coli*. These organisms appear to belong to the genus *Bdellovibrio* as originally described by Stolp and Petzold[6], and named by Stolp and Starr[7]. Typical newly formed plaques are shown in Fig. 3.

The fact that the lethal effect of seawater on *E. coli* increases (*a*) with increasing population size, as determined on a peptone–yeast extract agar, and (*b*) with an increasing number of lytic bacteria on inoculation with *E. coli* supports our assumption that the indigenous microflora contributes significantly to this effect. A more complex effect is suggested by the results of studies on other qualities of seawater, especially heavy concentration of metal and the presence of algal toxins. It seems reasonable to assume that the nature of the lethal effect of seawater on *E. coli* will vary with seasonal and local changes of the environmental conditions.

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G50. Lysis of *Escherichia coli* by Marine Microorganisms. RALPH MITCHELL. Harvard University, Cambridge, Mass.

The rapid die-off of *Escherichia coli* in seawater is as yet unexplained. Bacteria capable of lysing *E. coli* cell walls have been isolated from the sea. These bacteria killed *E. coli* in an artificial seawater medium. When the indigenous microflora was filtered from natural seawater, the rate of kill of *E. coli* declined noticeably. The return to filtered seawater of a high concentration of marine microorganisms, prepared by centrifugation, yielded much more rapid and complete killing of *E. coli* than natural seawater. A direct correlation between the size of the natural microbial population in the sea and the rate and degree of *E. coli* kill was detected. Repeated additions at 5-day intervals of *E. coli* to the same seawater sample resulted in greatly increased activity against *E. coli*. A high percentage of the bacteria following this treatment were capable of lysing *E. coli* cell walls. Preliminary experiments with a nonmarine fungus yielded similar results. These data suggest the development in seawater of a specific microflora which kills *E. coli*, and probably other nonmarine microorganisms, by enzymatically digesting their cell walls.

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Lysis of Non-Marine Fungi by Marine Micro-organisms

By R. MITCHELL AND C. WIRSEN

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(*Accepted for publication* 19 *January* 1968)

SUMMARY

A number of non-marine Oomycetes and a marine-isolated Pythium culture were lysed in natural sea water by marine micro-organisms. A relationship was observed between the rate and extent of lysis and the size of the marine microbial population. Microscopic observations and the accumulation of reducing sugars indicated that extracellular cell wall lysing enzymes were responsible. Pure cultures of lytic bacteria isolated from the sea yielded data similar to those obtained in natural sea water. The importance of the lytic phenomenon in the determination of the marine mycoflora is discussed.

INTRODUCTION

In the field of marine mycology it is necessary to investigate the biochemical activities of fungi in the oceans before we can understand their ecological significance and their distinct characters. The mycoflora of the oceans has not been studied in enough detail to identify marine-isolated fungi as specific halophiles, salt-adapted species, or non-marine fungi capable of survival in sea water (Johnson & Sparrow, 1961). MacLeod & Onofrey (1963) and ZoBell (1946) showed that some marine bacteria could develop the capacity to grow in the presence of reduced salt concentrations or even in freshwater media. Thus we may ask if some freshwater fungi adapt to marine conditions while others are eradicated by a biological process.

Previous data from our laboratory have shown that a specific marine microflora capable of killing *Escherichia coli* develops when that bacterium is added to sea water (Mitchell, Yankofsky & Jannasch, 1967). The presence in soil of micro-organisms capable of killing non-indigenous fungi by lysing their cell walls is well documented (Mitchell & Alexander, 1963). The present paper describes results obtained when a fungus whose habitat is soil was added to sea water. The fungus *Pythium debaryanum* was used because of the detailed information available about its cell-wall structure (Cooper & Aronson, 1967).

METHODS

In the current study the representative Phycomycete used was *Pythium debaryanum* ATCC 9998. Other Oomycetes used were *Saprolegnia diclina*, *Achlya caroliniania*, *Isoachlya luxuriana*, *Thraustotheca clavata* and *Apodachlya brachynema*. Fungal mats were grown in Petri dishes using a potato dextrose broth at 23°, and were washed with 200 ml. sterile water before use.

The sea water used was sampled at Woods Hole and at Nahant, Mass. When not used immediately it was stored at 3°. For pure culture studies artificial sea water (Lyman & Fleming, 1940) was used.

Marine bacteria were isolated on modified 2216E agar medium (Oppenheimer & ZoBell, 1952) and were grown in liquid 2216E medium in 250 ml. Erlenmeyer flasks at 23°.

Degradation studies of *Pythium debaryanum* in both natural sea water and by pure cultures were carried out in 250 ml. Erlenmeyer flasks at 25° on a new Brunswick (Model G 10) gyratory shaker. Mycelial mat weight was determined following collection of the fungus by filtration and drying at 110°, always in triplicate.

Cell-free filtrates were obtained after degradation of the mat in natural sea water by centrifuging for 10 min. at 7000 *g* and then aseptically filtering through 0·45 μ Millipore filters.

Some of the sea-water filtrate was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 are retained by the UM-1 ultrafiltration membrane. The apparatus (Diaflo Model 50 cell) and membranes were obtained from the Amicon Corp., Cambridge, Mass. Enzymic activity of the filtrates was tested against washed Pythium mycelium by assaying for release of reducing sugars by the anthrone method (Dische, 1955). Enrichment cultures were employed using Pythium cell walls prepared in a modified French Press (Raper & Hyatt, 1963).

RESULTS

Effect of the marine microflora on Pythium mycelium

In order to study the effect of the native marine microflora on Pythium mycelium, mycelial mats were added to sterile and non-sterile sea water. The mats remained intact in the sterile sea water, but were slowly degraded in static culture at 23° in fresh non-sterile sea water. Total degradation usually occurred 26 days after inoculation of the fungus.

The development of a specific antagonistic marine microflora was demonstrated by adding washed Pythium mycelial mats to fresh natural sea water, to autoclaved sea water, and to Millipore filtered (0·22 μ) sea water. The mycelia shaken in natural sea water completely disintegrated in 12 days, whereas in autoclaved and filtered sea water the mycelium was unaffected (Fig. 1).

When a second Pythium inoculum was placed in the flasks following degradation and subsequent removal of the remaining first mycelial mat, the second mat degraded, but much more rapidly, the time of degradation being 6 days (Fig. 1).

These data indicate either that the first Pythium inoculum stimulated the development of an antagonistic microflora which rapidly degraded the second inoculum of the fungus, or that an inducible enzyme system was stimulated.

Effect of pH, nutrients and dilution on the degradation of mycelium

Mycelial mats were placed in filter-sterilized sea water adjusted to pH values of 4, 5, 8 and 9. A loopful of inoculum from a Pythium culture being actively degraded in sea water was added to each flask. After 7 days the mats at pH 5, 8 and 9 showed signs of degradation, with the most extensive degradation occurring at pH 8. There was no degradation at pH 4 and the mats remained intact. It was observed that when 10 p.p.m. of 100 p.p.m. yeast extract was added to sea water along with a Pythium mat, the degradation process was accelerated. The process was accelerated even more when

yeast extract was added to the sea water 2 days before addition of the fungus, indicating that a stimulation of the native marine microbial population accelerated fungal lysis.

An indirect effect of bacterial population size was shown by dilution of the natural sea water. Sea water was sterilized by Millipore-filtration (0·22 μ). The undiluted samples contained 100 ml. natural sea water. The 1/10 dilution contained 90 ml. filter-sterilized sea water and 10 ml. natural sea water. The 1/100 dilution contained 99 ml. filter sterilized sea water and 1·0 ml. natural sea water. The time required for the degradation of fungal mats added to the flasks was followed by measuring mycelial mat weight. The mats in undiluted sea water displayed visible degradation after 4 days

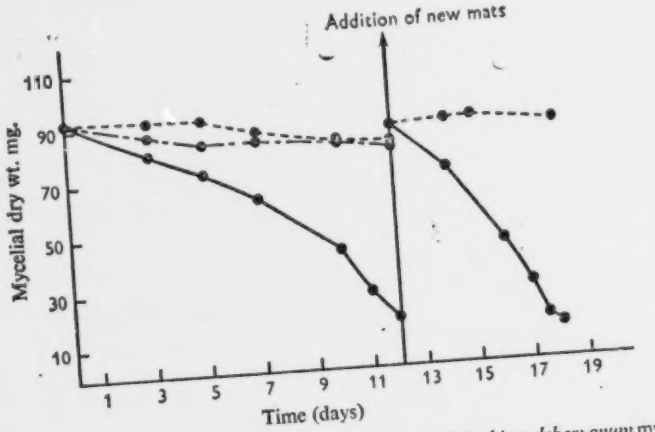

Fig. 1. Effect of repeated additions of mycelial mats on lysis of *Pythium debaryanum* mycelium in natural sea water. ——, Natural sea water, – – –, autoclaved sea water; — —, millipore-filtered (0·22 μ sea water).

and were totally degraded after 8 days; the mats in 1/10 dilution began to lyse after 7 days and were totally degraded after 12 days; the mats in the 1/100 dilution began to lyse after 9 days and were totally degraded after 17 days. These data provide further evidence that the degradation of Pythium is directly proportional to the size of the native marine microbial population.

Tests for enzymic activity

In subsequent experiments the ability of cell-free filtrates of sea water to degrade Pythium mycelium enzymically was tested. We used sea water which had been exposed to and had degraded two consecutive Pythium inocula so as to obtain maximal activity. When the second inoculum was completely degraded the sea water was centrifuged for 20 min. at 7000 *g* and filtered aseptically through 0·45 μ Millipore filters. Some of the sea water filtrate was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 were retained by the ultrafiltration membrane used in this study. Concentration of these molecules was achieved within approximately 1 hr in a single step using this method.

The concentrated and unconcentrated cell-free filtrates were tested for enzymic

activity against intact washed Pythium mycelium. The results obtained (Fig. 2) show that reducing sugars, measured as μg. glucose/ml., were released within 1 hr after addition of the mycelium to unconcentrated filtrate. The activity was substantially increased in the concentrated filtrate. The system was inactivated by heating the filtrate to 100° for 15 min. These data indicate the presence of extracellular lytic enzymes produced by marine micro-organisms which suppressed the non-marine fungus.

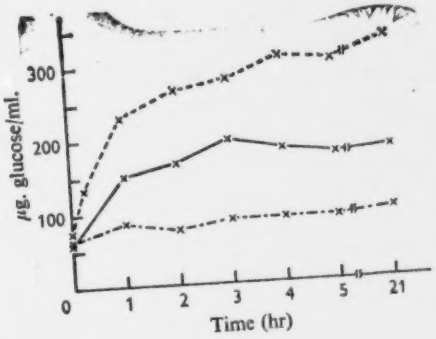

Fig. 2

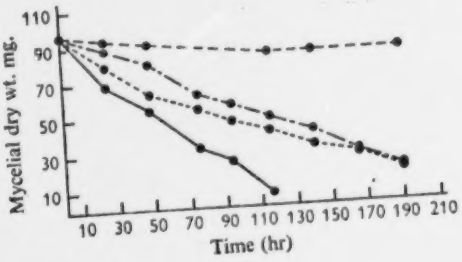

Fig. 3

Fig. 2. Release of reducing sugars during lysis of *Pythium debaryanum* mycelium by filtered sea water. The sea water was subjected to repeated inoculation with Pythium before filtration. ——, Unconcentrated filtrate; – – –, concentrated (11·5×) filtrate; —·—, boiled filtrate.

Fig. 3. Relationship between the numbers of lytic bacteria present and the rate of lysis of *Pythium debaryanum*. – – –, Autoclaved sea water; – — –, sea water and 10^4 bacteria/ml. added; - - -, sea water and 10^5 bacteria/ml. added; ——, sea water and 10^6 bacteria/ml. added.

Pure culture studies

Isolations of marine bacteria were made from sea water to 2216E media following degradation of a mycelial mat. A bacterium, tentatively identified as an *Agarbacterium* sp. which would degrade Pythium in pure culture, was isolated from sea water enriched with Pythium cell walls. This bacterium yielded results similar to those observed in natural sea water. However, the degradation time was more rapid in pure culture. Fig. 3 shows data indicating that when the cell concentration of *Agarbacterium* sp. added to artificial sea water was 10^4/ml.; the fungal mat was degraded in 190 hr. When the concentration was 10^6/ml., degradation occurred in 116 hr. A higher level of antifungal activity was detected following repeated inoculation of the fungus. When a fungal mat was added to artificial sea water inoculated with 10^6/ml. *Agarbacterium* sp. the mat was degraded in 115 hr, whereas the second inoculum of the fungus was degraded by the bacteria in 75 hr (Fig. 4). Many spheroplasts of the fungus were observed under phase-contrast microscopy during the course of degradation, suggesting that lysis of the fungal cell walls was occurring.

Comparison of lysis of a marine isolated Pythium with Pythium debaryanum

Mycelial mats of *Pythium debaryanum* grown in potato dextrose broth were washed and added to flasks containing 100 ml. autoclaved sea water and to flasks containing

100 ml. of natural sea water. The same procedure was followed for a marine isolate of Pythium. The marine species was grown in potato dextrose broth prepared with 75% sea water. Mycelial mat weights were determined, in triplicate, during degradation. The results presented in Fig. 5 show that in the time period tested the mycelial stage of the marine isolate was as susceptible to degradation by the marine microflora as *Pythium debaryanum*. This may imply salt adaptation on the part of the marine isolate with no or very slight changes in its cell-wall structure, thus giving it no competitive advantage over the freshwater strain.

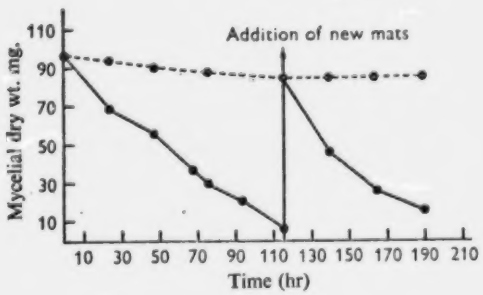

Fig. 4

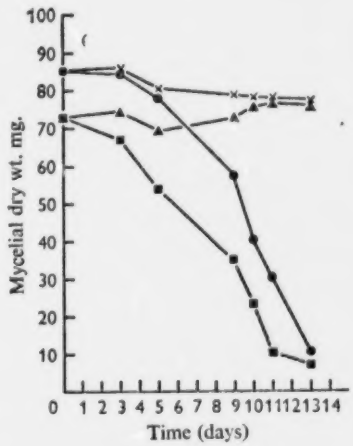

Fig. 5

Fig. 4. The effect of repeated additions of mycelium on degradation of *Pythium debaryanum* by a lytic bacterium isolated from the sea. – – –, Autoclaved sea water and 10^6 bacteria/ml. added.

Fig. 5. A comparison of the rates of lysis in natural sea water of a freshwater strain and a marine isolated Pythium strain. ▲—▲, Salt water Pythium control; ■—■, salt-water Pythium test; × — ×, freshwater Pythium control; ●—●, freshwater Pythium test.

The effect of salt adaptation on the susceptibility to lysis of the non-marine Pythium was tested by first adapting *Pythium debaryanum* to grow in potato dextrose broth prepared in 80% artificial sea water. This was achieved by a series of serial transfers to 10, 20, 40, 60, 70 and 80% artificial sea water. The fungus was then tested for resistance to degradation. It was observed that the salt-adapted *Pythium debaryanum* was degraded as rapidly as the non-adapted *P. debaryanum* both in natural sea water and in artificial sea water with 10^6/ml. *Agarbacterium* sp. added.

Degradation studies of other Oomycetes

Five isolates of closely related Oomycetes were tested for resistance to degradation in natural sea water and also to degradation by the Pythium degrading bacterium. The isolates were *Apodachlya brachynema*, *Saprolegnia diclina*, *Achlya caroliniana*, *Isoachlya luxuriana* and *Thraustotheca clavata*. Mycelial mats of these fungi were grown in potato dextrose broth, collected, washed, and added to 100 ml. natural sea water and to 100 ml. autoclaved sea water in 250 ml. Erlenmeyer flasks. All of the mats in natural sea water turned yellowish and were entirely degraded in no more than 8 days

in shake culture. *Apodachlya brachynema* was degraded in 8 days; *S. diclina* in 5 days, *A. caroliniania* in 7 days, *I. luxuriana* in 6 days, and *T. clavata* in 5 days. The mats in sterile sea water remained white, perfectly intact and viable.

These five fungi were then tested with the *Agarbacterium* sp. previously found capable of decomposing Pythium in sterile sea water. Washed mycelial mats were added to sterile sea water and an inoculum of 10^6 bacteria/ml. was added. All five fungi were susceptible to lysis by this bacterium in no longer than 5 days. *Traustotheca clavata*, *Saprolognia diclina* and *Apodachlya brachynema* were completely degraded in 3 days; *Isoachlya luxuriana* in 4 days; and *Achlya caroliniania* in 5 days.

Thus it appears that there is a specific microflora which attacks the mycelial stage of these fungi in sea water. The same bacterial strain isolated from sea water was active against all of the fungi tested. These results indicate a close similarity in cell-wall structures of these fungi.

DISCUSSION

Large numbers of fungi have been isolated from the sea (Johnson & Sparrow, 1961). We do not know if all these fungi grow actively in the sea, or if the isolates simply reflect survival of these fungi in the form of spores. We have observed that when *Escherichia coli* is added to sea water, an antagonistic marine microflora develops which kills that bacterium (Mitchell, Yankofsky & Jannasch, 1967). In the current investigation we present evidence that an antagonistic marine microflora also develops in response to the addition to sea water of the mycelial form of several Oomycetes.

The observation that the population size of the marine microflora affects the rate of kill of the fungus provides further evidence indicating the implication of a group of native marine micro-organisms in the eradication of *Pythium debaryanum* in the sea. The data showing that the rate of lysis of Pythium mycelium increases with each subsequent addition of the fungus to the same sample of sea water suggest the enrichment of a specific antagonistic group of micro-organisms or the increase in adaptive enzyme capacity or both.

We have reported previously the enzymic lysis of *Pythium debaryanum* in soil (Mitchell & Hurwitz, 1965). It was not surprising, therefore, to find that culture filtrates of sea water contained enzymes capable of lysing Pythium following exposure of the sea water to mycelium of that fungus. We did not expect, however, that such large quantities of enzyme would be active in the sea water. Both enzymes (Mitchell & Nevo, 1965) and Bdellovibrios (Mitchell *et al.* 1967) have been associated with the killing of *Escherichia coli* in sea water. Apparently, enzymic lysis of the cell walls is solely responsible for the decline of Pythium.

A similar susceptibility to lysis was detected when other non-marine Oomycetes were added to sea water. The observation that a marine isolated Pythium strain was equally susceptible to lysis suggests that fungi isolated from marine estuaries may not in fact, be ecologically restricted to the marine environment. Resistance to lysis by a marine microflora may be a useful tool in the characterization of a true mycoflora.

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Factors affecting the decline of non-marine microorganisms in seawater

A Review

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INTRODUCTION

Each year the quantity of sewage entering the oceans from sewage outfalls increases. We have always assumed that the sea has an inherent capacity to dispose of intestinal pathogens carried in sewage. In recent years, in many estuaries, the volume of sewage flowing into the sea has overcome the capacity of the marine environment to purify itself and it has become increasingly important to understand the mechanisms by which the sea eradicates non-marine microorganisms flowing into it.

Fungi, bacteria and viruses pathogenic to man are carried by sewage into the sea and are potential sources of disease. There is a large and controversial literature proposing mechanisms by which enteric bacteria are killed in the sea. As early as 1889 DeGiaxa noted that bacteria survive to a greater extent in heat sterilized seawater than in untreated seawater, and in 1936 ZoBell noted that natural seawater had a bactericidal action on non-marine bacteria. Vaccaro et al (1950), in a study of the viability of Escherichia coli in seawater, showed that the survival of that organism in the sea varied with the season. E. coli declined more rapidly during the summer months.

Ketchum, Ayres and Vaccaro (1952) studied the significance of dilution and the bactericidal action of seawater on the survival of coliforms in esturine waters. They found that most of the decline in numbers of coliform bacteria was accounted for by a bactericidal action of the water and that dilution appeared to play a small part in the kill. They concluded that a biologically produced "antibiotic" was involved. The survival of enteric organisms in seawater was reviewed by Greenberg, (1956). He also concluded that the disappearance of fecal bacteria from marine estuaries is greater than that which would be expected from dilution alone and that a number of different factors are involved in the decline. These include the production by marine

bacteria of unidentified heat labile antibiotic substances, adsorption, sedimentation, predation, and competition for a limited nutrient supply. We will concern ourselves in this review mainly with the literature since 1956, and especially with the mechanisms by which enteric microorganisms are killed in the sea.

BACTERIA

A typical die-off curve of Escherichia coli in seawater shows an initial lag phase followed by a mortality of up to 90% in three to five days. Orlab (1956), showed that a typical killing curve of E. coli had a lag phase, a phase of rapid decline, a phase in which resistant organisms developed, and ultimately a phase in which the coliforms grew back again. He presented data indicating that the bactericidal agent in seawater was biological and heat labile. The bactericidal factor was diminished by boiling, autoclaving, filtration, pasteurization and chlorination. Orlab suggests that sedimentation and adsorption are significant factors in the removal of intestinal bacteria from the sea, and that the presence of organic matter in the estuary off-sets die-off by stimulating bacterial growth. Increased temperature has been found to increase the rate of decline and to shorten the lag phase. This temperature effect would explain why a more rapid kill is observed during the summer months.

The survival of the enteric pathogenic bacterium Shigella in seawater was studied by Nakamura et al (1964). They found a greater reduction in numbers of Shigella in natural than in autoclaved or filter-sterilized seawater, and expressed the belief that this increased kill in natural seawater resulted from the production of biological toxins, since filtration of microorganisms from the water allowed Shigella to survive longer. Further support for the presence of a biologically produced toxin in seawater is indicated by the work of Jones, (1963). Using a number of different non-marine bacteria, he demonstrated that natural seawater was more inhibitory than artificial seawater.

Storage of the water at 4°C resulted in a loss of inhibitory activity. Jones concluded that this may result from adsorption of toxic materials onto the glass storage containers. A number of different biological factors have been considered to control the survival of Escherichia coli in sea water. Pramer and his co-workers have carried out a detailed study to evaluate these different factors. Carlucci and Pramer (1960), presented data indicating that antibiotics were not a significant factor in the decline of Escherichia coli in seawater. They tested 200 isolates of marine bacteria for antibiotic activity against either E. coli or Bacillus subtilis, without detecting any significant activity. Penicillin and chlortetracycline were rapidly inactivated in both sterile and natural seawater.

In a later study Carlucci and Pramer, (1960a) showed that marine bacteriophages had no effect on the survival of Escherichia coli in the sea. They used heat and filter sterilized seawater in an attempt to evaluate the significance of non-biological factors on the survival of E. coli in seawater, and found that the intestinal bacterium survived better in filtered than in natural water (Carlucci, Scapino and Pramer, 1961). They concluded that predators and competitors had been removed by filtration. Autoclaving removed an antagonistic factor found in filter-sterilized sea water. The authors suggested that a heat labile toxin was removed by autoclaving, or that nutrients were released. In a survey of their research in this area, Pramer, Carlucci, and Scarpino, (1963), postulate that the significance of biological factors in the killing of Escherichia coli in sea water had been over-emphasized and propose that greater attention be placed on the effect of temperature and physico -chemical characteristics of seawater. They provided evidence for a physiochemical killing process in the sea, by showing that artificial seawater was as bactericidal

as natural seawater. However, they were not able to conclude that the same factors were responsible for the killing of bacteria in natural and artificial seawater. Both Carlucci and Pramer (1960) and Johanneson (1957) found that the addition of cysteine increased the survival of E. coli in the water. The nature of this cysteine effect is not known. However, it does indicate that, at least under certain conditions, a competition for nutrients may be responsible for the decline of non-marine microorganisms in the sea.

The possibility that heavy metals are an important factor in the killing of E. coli in seawater has been considered by Jones (1963). He found that Escherichia coli was inhibited in filter sterilized, artificial, or natural seawater. This inhibition was reversed by autoclaving the water. The addition of chelating agents could substitute for autoclaving in the toxicity of filter sterilized seawater, and led to the conclusion that the toxicity probably originates in the trace quantities of heavy metals. He reported that with E. coli in filter-sterilized seawater supplemented with glucose, ammonium chloride, and potassium phosphate growth was stimulated by cysteine, case in hydrolyate peptone, or autoclaving. Chelating agents had similar stimulating effects. The author concluded that the toxicity was caused by heavy metals, and that these metals are chelated by autoclaving the water. Jones' data indicate the process which inhibits growth of E. coli in seawater. However, this inhibition of growth is not necessarily applicable to the killing of E. coli in seawater. Cells which do not grow in a medium may remain viable in that medium for some time. E. coli does remain viable in filtered seawater, but is rapidly killed in natural seawater, indicating the presence of an agent more actively antagonistic than the inhibitory heavy metals.

The possibility that intestinal microorganisms carried into the sea are rapidly sedimented in a manner similar to flocculation in sewage treatment was considered by Rittenberg et al (1958). They surveyed the distribution 23

of coliforms in sediments around three marine sewage outfalls, and found that large quantities of coliforms were deposited below the outfall. They suggest that the bacterial cells released in the effluents flocculate and settle out in the seawater.

Despite the conclusions of Pramer and his co-workers that the decline of E. coli in seawater is caused primarily by a physio-chemical process, a number of workers have attempted to implicate the marine microflora. We would expect that with the wide variety of microorganisms present in the sea there would be a large number of bacteria and actinomycetes present which would be antagonistic to other microorganisms. Rosenfeld and ZoBell, (1947), tested 58 species of marine microorganisms and found that nine of them were antagonistic to non-marine microorganisms and they assumed that the antagonistic material was an antibiotic. Krassnilnikova (1962), detected antibiotic production by large numbers of microorganisms isolated from the ocean. Despite the large number of antagonists isolated, cultivation of these marine organisms in natural seawater resulted in little if any antimicrobial activity.

It should be pointed out here that there is no real connection between the isolation of antibiotic producing microorganisms and the production of antibiotic under natural ecological conditions. It seems likely that Pramer's conclusion that antibiotic production is not a significant factor in antagonisms in the ocean is a correct one. An analogous situation exists in the soil. Numerous investigators have attempted to detect antibiotic production in the soil. However, no such production has ever been detected, despite the fact that virtually all antibiotics used in chemotherapy have been isolated as products of soil microorganisms. All of these soil microorganisms are cultured for antibiotic production in rich media under highly defined conditions.

When these organisms are placed in natural soil little or no antibiotics are produced. We would expect a similar condition to exist in the sea.

Very large populations of unicellular algae develop in the presence of increased organic matter concentrations found in polluted seawater. It would seem logical that under certain circumstances these algae might be involved in the killing of microorganisms carried in the sewage. Laboratory studies of factors affecting the kill of non-marine microorganisms in seawater are normally carried out in the dark. Algal toxins would not be produced under these conditions, so that the effect of algae would not be taken into account. A number of years ago Voroshilova and Dianova (1937) found that marine plankton reduced the bacterial population in samples of seawater. Recent studies have indicated that antagonistic materials produced by marine algae may be important in marine ecology. Duff et al (1966) in a study of the anti-bacterial activity of marine planktonic algae found that extracts of marine plankton were active against both marine bacteria and gram positive non-marine bacteria, including Staphylococcus. Antibiotic activity by marine algae has been demonstrated by Burkholder et al, (1960). They tested 150 marine algae for activity against Staphylococcus aureus, and found that 66 isolates displayed activity against that pathogen. In a later study Olson et al, (1964) showed that a number of different algae inhibited growth of S. aureus and the pathogenic yeast Candida albicans. They detected at least six different antimicrobial substances produced by these algae.

Evidence for the production of toxins by plankton which may be responsible for the bactericidal effect of seawater on sewage has been provided by Aubert (1964). He found that the antagonistic materials produced by plankton were thermolabile. The nature of these antagonistic materials produced by unicellular algae has been studied by a number of workers. Different algae have been extracted with organic solvents by Jorgensen(1962). He found that alcohol

and ether extracts of cells of Chlorella, Scendesmus and Chlamydomonas inhibited Bacillus subtilis, and associated the inhibition with two substances. One was a chlorophyllide associated with the algal chlorophyll, and only showed activity after illumination. The second substance was not associated with the pigment. Evidence was obtained indicating that these substances produced by the algae play a part in the inhibition of bacteria in natural waters.

Sieburth (1960) isolated and characterized an "antibiotic" produced by the mucilagenous alga Phaeocystis. He identified the material as acrylic acid and demonstrated that concentrates of this alga gathered from the sea inhibited a wide range of pathogenic bacteria. Sieburth and Pratt (1962), in a study of the effect of algal blooms on Escherichia coli in natural seawaters, found that samples of seawater containing the alga Skeletonema costatum inhibited Escherichia coli. They were of the opinion that phytoplankton may have widespread significance in killing non-marine bacteria in the sea. A report by Nielson (1955) demonstrated that marine plankton produce "antibiotics" in the sea and that these "antibiotic" materials are free in the water. He found that the presence of unicellular algae reduced the number of bacteria in the sea. Saz et al, (1963) have studied the bactericidal action of seawater against Staphylococcus and have demonstrated that the active factor is a large non-dialyzable heat-labile molecule. They speculate that the production of this antagonistic material may be associated with phytoplankton blooms. It is apparent that algae proliferate in the presence of the nutrients provided by sewage. Some of these algae have the capacity of excreting antibacterial toxins, and under certain circumstances algae may be implicated in the decline of intestinal bacteria in the sea.

The lysis of non-marine microorganisms by marine bacteria has been studied by the author and his co-workers. Mitchell and Nevo (1965) isolated bacteria

from the sea which were capable of killing Escherichia coli in artificial seawater by enzymatically degrading the cell walls. In a later study Mitchell, Yankofsky and Jannasch (1967) studied the effect of the native marine microflora on the killing of Escherichia coli in natural seawater. They found that the rate of kill of Escherichia coli in natural seawater was proportional to the size of the marine microflora, and provided evidence for a direct relationship between the activities of microorganisms in the sea and the rate of kill of E. coli in that environment. Two groups of microorganisms were associated with the kill: (a) cell wall lysing bacteria and (b) a group of marine parasitic bacteria similar to Bdellovibrio bacteriovorus originally described by Stolp and Starr, (1963). This latter group of extraordinarily interesting bacteria have been isolated frequently from soil and sewage. They are capable of passing through a 0.45μ pore size filter and are typically parasitic, although saprophytic mutants have been isolated. The hosts apparently are exclusively gram negative bacteria, although it would appear to be only a matter of time before other hosts are detected. They appear to be present in large numbers in the sea. It would be interesting to determine their ecological significance in the decline of enteric bacteria in sewage treatment plants or in rivers with sewage outfalls. In a later study Mitchell (unpublished results) showed that protozoa are also involved in killing E. coli in seawater. The protozoa develop slowly in relation to the marine bacteria. However, when seawater was continually inoculated with E. coli protozoa ultimately dominated the medium, and all other microorganisms, both coliforms and marine bacteria were rapidly digested. Marine nematodes have also been found to be capable of digesting coliforms. However, the rate of decline of E. coli in seawater to which marine nematodes have been added is much slower than that normally observed when microorganisms are added. Usually a 50% to 70% decline in E. coli was observed in approximately 10 to 12 days compared to a 90% decline in 5 days in seawater with a high concentration of other microorganisms.

The chemostat would appear to be an obvious instrument to study the mechanism of decline of non-marine microorganisms in the sea. Batch cultures present an artificial picture and a kinetic approach would be more realistic. In a recent paper, Jannasch (1968) has used the chemostat to study the kinetics of mixed populations in marine systems. He showed that when the concentration of carbon and energy sources was limiting at low dilution rates the native marine microflora competitively displaced E. coli from seawater. It appears that under the growth limiting conditions in the sea the non-marine microorganisms are not capable of competing with the marine microflora.

The bactericidal action of seawater varies with the location, sampling time, and a variety of other factors. It is clear that many different parameters may be associated with the kill of non-marine microorganisms in the sea. Processes may be implicated in tropical waters which would have no relevance to temperate waters. Extracts of Gorgonian corals have been found to be active against E. coli, Micrococcus, Clostridium, and the pathogenic yeast Candida albicans by Burkholder and Burkholder, (1958). The antimicrobial activity of corals has been associated with terpenoid lactones by Ciereszko (1962). Ethyl ether extracts of red-beard sponges have been found to have a bactericidal effect on gram positive, gram negative and acid fast bacteria as well as Candida albicans by Nigrelli, Jakowska, and Calventi (1959). There is also a possibility that molluscs may be involved under certain circumstances in the killing of non-marine microorganisms in the sea. Li, et al (1962) isolated agents active against Streptococcus pyogenes, Staphyloccus aureus, poeiomyelitis virus and influenza virus from molluscs. It should be noted that most of these antagonistic materials were obtained by extracting the cellular material with organic solvents. While this may be a useful technique in the search for new chemotherapeutic agents, the lack

of contact between the antagonistic material and the non-marine microflora would seem to make it unlikely that these materials are responsible for the killing of those non-marine microorganisms in the sea.

Yeasts and Fungi

A number of yeasts and fungi pathogenic to man are carried in sewage, and are a potential source of disease in outfalls where sewage is disposed. We know very little about the survival of non-marine yeasts and fungi in seawater. Dabrowa et al (1964), carried out a survey of tide washed coastal areas of Southern California and found a number of fungi pathogenic to man. Yeasts normally found in the human intestine have been detected by van Uden and Branco (1963), in the guts of seagulls living in warm tropical water. Taysi and van Uden, (1964), could not detect any intestinal yeasts in temperate estuaries of Portugese rivers. They believe that temperature is the important ecological factor in the killing of yeasts carried into estuaries in sewage, since most intestinal yeasts require temperatures for growth which are substantially higher than those found in temperate waters. Seawater and sodium chloride was found to have an antagonistic effect on the growth of fungi pathogenic to man by Dzawachiszwilli et al, 1964. The nature of the anti-yeast and anti-fungal activity of seawater has been investigated by a number of workers. Buck et al, (1962) tested 132 isolates of marine bacteria for anti-yeast activity and showed that culture filtrates of a number of these bacteria had strong activity. In a later paper Buck et al (1963) reported the isolation of a Pseudomonas species from a tube-dwelling amphipod community which strongly inhibited the pathogenic yeast Cryptococcus neoformans. Buck and Myers (1965) tested the activity of marine pelagic plankton, sponges, and alcyonarians against pahtogenic yeasts. Very little antagonistic activity was detected. However, approximately 8% of the marine bacteria isolated

from amphipods were found to inhibit non-marine yeasts including Candida albicans. They are of the opinion that the activity of these anti-yeast bacteria in the sea may be significant in the natural biological control of both pathogenic and non-pathogenic yeasts entering estuaries from terrestial sources.

Welch (1962), surveyed the fungistic properties of a number of marine algae. She homogenized preparations of 35 marine algae and tested their activity against 6 pathogenic fungi, and found that extracts of 11 different algae strongly antagonized one or more of the fungi or yeasts tested indluding Candida albicans and Histoplasma capsulatum.

Mitchell and Wirsen (1968), have investigated the effect of the native marine microflora on the killing of fungi in seawater. They found that a specific antagonistic microflora was developed in response to the inoculation of a non-marine fungus into natural seawater. The predominant microorganisms were bacteria they and apparently killed the non-marine fungus by enzymatically lysing the cell walls. It seems likely that a number of different mechanisms are involved in the killing of non-marine fungi and yeasts in the sea but the nature of these antagonistic factors is still obscure.

Enteric Viruses

The importance of enteric viruses transmitted by sewage in seawater is difficult to assess. The comsumption of improperly cooked or raw shell fish harvested from polluted seawater is responsible for a series of epidemics of infectious hepatitis. The virus appears to be taken up by the shell fish and survives in the shell fish tissue. Similarly, poliomyelitis virus and coxsackie virus probably are transmitted to shell fish from polluted seawater.

We know surprisingly little about the effect of seawater on the survival of animal viruses. Metcalf and Stilles, (1967) have studied the survival of enteric viruses in estuarine waters. The viruses survived up to 30 days during the summer and up to 55 days during the winter in the water. When natural seawater was compared with seawater polluted with domestic sewage, it was interesting to note that the sewage had a protective effect on the virus, increasing the survival time from 14 to 35 days. The viruses were protected for an even longer period of time in oysters. In the light of our earlier discussion of the bactericidal action of marine microorganisms it would seem reasonable that in the polluted seawater the native microflora has been displaced so that any possible inactivation of the virus by marine microorganisms would not be possible. Liu et al, (1967) have demonstrated that when shell fish containing viruses are placed in clean natural seawater, they purify themselves of viruses. They presented data indicating that the virus in the digestive system of the shell fish was not absorbed onto nor did it penetrate the fish cells. They indicate that pollution of shell fish is a dynamic process and that placing the shell fish in clean seawater allowed it to purify itself by dilution. Many questions remain to be answered with respect to the survival of viruses in seawater. It would be interesting to know if there are anti-viral toxins produced in seawater, if enzymes capable of degrading viruses are produced by marine microorganisms or if some other factors are involved in the killing of viruses in the sea.

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ABSTRACT OF PAPER TO BE PRESENTED AT THE

INTERNATIONAL WATER POLLUTION RESEARCH

CONFERENCE, PRAGUE 1968.

REPRODUCED FROM ''WATER RESEARCH''

MARCH 1968

Lysis of intestinal and other non-marine micro-organisms by marine bacteria. R. MITCHELL, Cambridge, Massachusetts, U.S.A.

Surprisingly little is known about the mechanism by which intestinal and other non-marine micro-organisms die in the sea, despite the increasing pollution of the marine environment. We have been concerned in our laboratory with the contribution of the indigenous marine microflora to the killing of intestinal bacteria, represented by *Escherichia coli* and of fungi represented by *Pythium debaryanum*.

Initially, the relationship between the size of the marine microbial population and the rate of *E. coli* die-off was studied. Large quantities of marine micro-organisms were collected by centrifugation of fresh sea water. These micro-organisms were added at different concentrations to autoclaved sea water in 250 ml Erlenmeyer flasks. *E. coli* was inoculated and the flasks were incubated at 30° C on a rotary shaker. Counts of *E. coli* were made at regular intervals on eosin methylene blue agar plates incubated at 37° C. The results obtained (FIG. 1) show that there is a direct relationship between the size of the marine microflora and the rate and extent of die-off of *E. coli* in sea water.

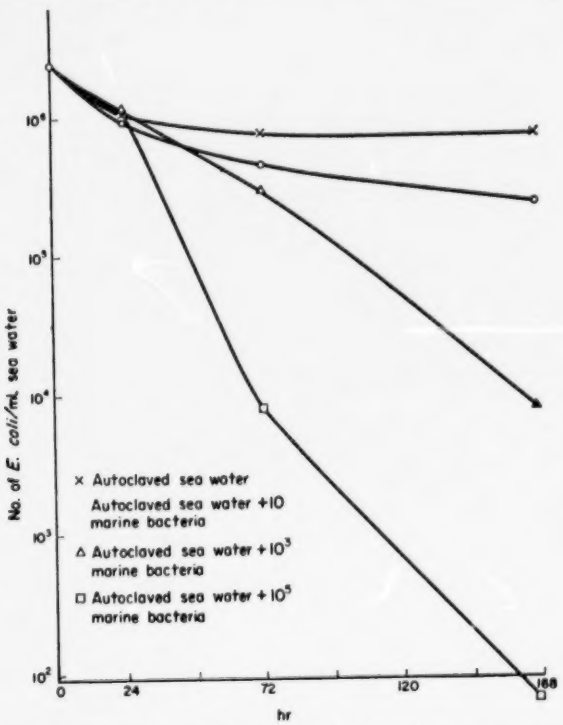

FIG. 1. Relationship between the number of bacteria in a sample of sea water and the rate and extent of die-off of *Escherichia coli*.

filters. The filtered sea water which was found to be sterile was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 were retained by the ultrafiltration membrane. Concentration was achieved within approximately 1 hr in a single step without the need for dialysis using this method.

These concentrated cell free filtrates were tested for enzymatic activity against whole washed *Pythium* mycelium. *Pythium* cell walls contain a high percentage of glucans so activity was measured by assaying for release of reducing sugars.

The results obtained (FIG. 2) show that reducing sugars were released from the mycelium within 1 hr after the addition of filtered sea water. The enzymatic activity was substantially increased following tenfold concentration of the materials having a molecular weight higher than 10,000. The enzymes were inactivated by heating to 100° C for 20 min. This study points to the importance of enzymes produced by marine micro-organisms in suppressing non-marine micro-organisms in the sea. The enzymes involved in the lysis are still unknown. It seems reasonable to suppose that more than one enzyme is involved. Further information on the mechanism of this lytic process must await isolation of the responsible micro-organisms.

The fate of viruses in a marine environment. YEHUDA KNOTT, HANNA BEN ARI and NETTY BURAS, Haifa, Israel

In recent years, due to increased industrialization and to the steady flow of population from under-developed areas to large urban centres, there has been ever-growing pollution of the sea coast (as well as of the sea) by domestic and industrial wastes. The decision as to whether treated or untreated sewage should be discharged into the sea is based in many cases solely on local considerations. The problems raised by this pollution have motivated a number of studies, some of them presented at the First Symposium on Marine Pollution (PEARSON, 1966).

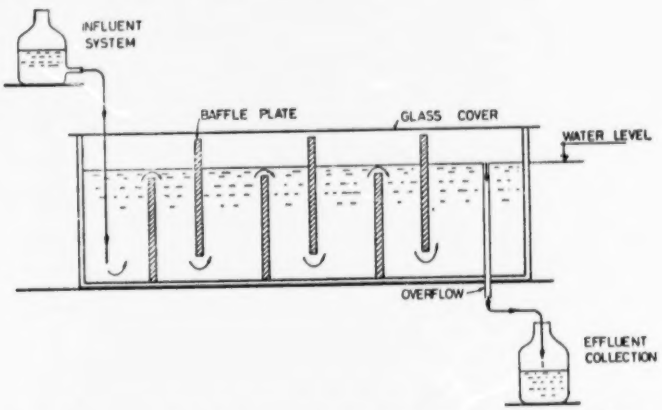

FIG. 1. Schematic flow diagram of sewage–sea water mixture in aquarium.

The conventional criterion in estimating pollution due to domestic sewage is the coliform index. The suitability of *Streptococcus fecalis* and other bacteria as indicators of marine pollution has also been studied, but the techniques necessary were found to be too complicated and expensive. Some of the studies dealt with sea currents governing the drifting of domestic and industrial waste (EGGINK, 1967), others with dilution and dispersion due to tides (HARLEMAN, 1967); they were based on the use of bacteria and viruses (SINER and PINOL, 1967), or of tracer dyes like Rhodamine B or fluorescein (WALDICHUK, 1967).

Little information is available on the dynamics of purification by dilution. Of the prominent studies on this subject, mention should be made of the possible action of antibiotics produced by marine algae (AUBERT and GAUTHIER, 1966). The controversy as to the reliability of the coliform index has

The development of a specific antagonistic microbial population following inoculation of *E. coli* into sea water was indicated in experiments in which *E. coli* was re-inoculated to natural sea water 5 days after the initial inoculum. The results show that the lag period in the die-off of *E. coli* was virtually eliminated and the slope of the die-off curve was significantly sharper than that observed with the first inoculum.

In a parallel study, the survival of *E. coli* was investigated in sea water samples taken from the Atlantic Ocean 150 miles off Cape Cod, Massachussets. *E. coli* populations declined from 10^9/ml to 10^7/ml in 5 days in those samples in which the total bacterial population approximated 10^3 bacteria/ml, as determined by plate counts. By comparison, the *E. coli* population declined from 10^9/ml to 10^4/ml in 5 days in sea water sampled from a harbor at Woods Hole, Massachussets. The harbour was approximately 10^6/ml. Thus it appears that the rate of kill of *E. coli* under natural conditions in the sea is at least partially controlled by the size of the native marine microbial population.

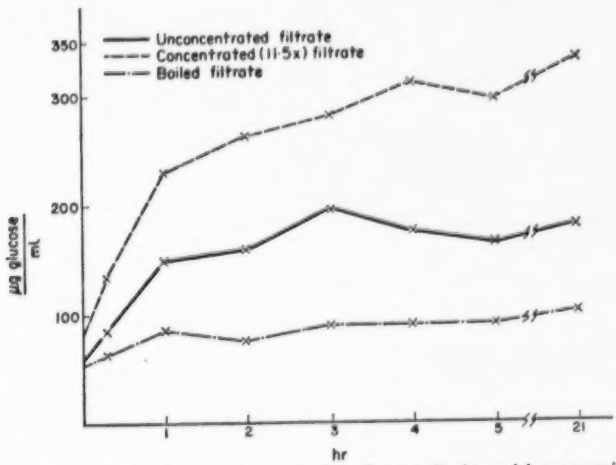

FIG. 2. Release of reducing sugars during lysis of the fungus, *Pythium debaryanum* in sea water filtrates.

Phase contrast microscopy revealed the presence of large numbers of vibrios in filtrates following exposure to *E. coli*. Very few filterable vibrios were present in natural sea water before exposure to *E. coli*. The vibrios varied in size from approximately 0·2 μ to 1·0 μ long and were highly motile. They yielded plaques in 3 days when sea water containing them were added to double layer plates containing *E. coli* and autoclaved sea water in 0·7 agar and incubated at 30° C. Vibrios strongly resembled the parasitic filterable bacterium *Bdellvibrio bacteriovorus*.

Our evidence suggests that a microbial population capable of lysing *E. coli* is present in the sea. It appears that a large population of these parasitic bacteria develops in sea water contaminated with *E. coli*. Whether these bacteria alone are responsible for the killing of *E. coli* and other Gram negative bacteria, or if they act together with other micro-organisms in the sea remains to be determined.

When fungal mycelium was added to natural sea water it completely disintegrated in 12 days whereas in sterile sea water the mycelium was unaffected. Thus, it appears that there is also a native marine microflora capable of degrading this fungus. Evidence for this hypothesis was strengthened by results obtained when a second inoculum of *Pythium* was placed in the Erlenmeyer flask following disintegration of the first mycelial mat. The second inoculum was complete in 6 days. These data indicate that the inoculum of *Pythium* stimulated the development of an antagonistic microflora which rapidly disintegrated the second inoculum of the fungus.

In subsequent experiments the ability of cell-face filtrates of sea water to enzymatically degrade whole *Pythium* mycelium and fungal cell walls was tested. We used sea water which had been exposed to and had degraded two consecutive inocula of *Pythium* so as to obtain maximal activity. When the second inoculum was completely degraded the sea water was filtered through 0·45 pore size Millipore

Running head

LYSIS OF NON-MARINE MICROORGANISMS BY MARINE BACTERIA

GALLEY PROOF

SARSIA—MITCHELL—1

THE EFFECT OF WATER MOVEMENT ON LYSIS OF NON-MARINE MICROORGANISMS BY MARINE BACTERIA

By

Ralph Mitchell

~~Laboratory of Applied Microbiology,~~
Division of Engineering and Applied Physics, Harvard University, Cambridge, Massachusetts, U. S. A.

ABSTRACT

It has been demonstrated that the kill of non-marine microorganisms in the sea is directly related to the size of the marine microbial population. In the deep sea where the microorganisms are sparse there is a minimal effect on non-marine microorganisms. However, water sampled from coastal zones had a strong killing effect.

Large numbers of non-marine microorganisms are carried into the sea in sewage and in surface drainage waters. These microorganisms, which are foreign to the marine environment, rapidly disappear in the sea. Hypotheses proposed to account for this decline include dilution by ocean currents (Vaccaro et al. 1950), physico-chemical factors (Pramer et al. 1963), and toxic products of marine microorganisms (Jones 1963). We have been concerned in our laboratory with the contribution of the indigenous marine microflora on the killing of intestinal bacteria carried into the sea in sewage, represented by *Escherichia coli*, and of soil fungi, represented by *Pythium debaryanum*.

In an earlier study we isolated bacteria capable of enzymatically degrading *E. coli* from the sea (Mitchell & Nevo 1965). These marine bacteria killed *E. coli* when added to artificial sea water. In the current study we have investigated the contribution of the indigenous marine microflora in polluted areas and in the open sea to the killing of non-marine microorganisms.

METHODS

Sea water was sampled at Woods Hole, Massachusetts, at Nahant, Massachusetts, and at a distance of 150 miles due east off the coast of Massachusetts. The water was stored at 3°C for not longer than 4 weeks before use. Cultures of *E. coli* and *Pythium* were incubated in natural sea water at 25°C on a New Brunswick rotary shaker. *Escherichia coli* was counted on eosin methylene blue plates following incubation at 37°C, Counts of marine bacteria were made on Oppenheimer & Zobell's (1952) medium following incubation at 25°C. *Bdellovibrio bacteriovorus* was isolated following three repeated inoculations of sea water with *E. coli* at 5-day intervals. The sea water was then filtered through a Millipore filter with a pore size of 0.45 μ. The filtered water was plated on double layer plates. The lower layer consisted of sterile sea water with 1.5% agar added. The top layer comprised sterile sea water with a heavy suspension of washed *E. coli* added, and solidified with 0.7% agar. Clearing zones caused by growth of *Bdellovibrio* in the opaque *E. coli* layer appeared following 5—7 days incubation at 25°C.

The fungus used was *Pythium debaryanum* ATCC—9998. Fungal mats were grown in potato dextrose broth (MITCHELL & HURWITZ 1965) in petri dishes, and were washed with 200 ml sterile water before inoculation into sea water. Degradation of the fungus was determined by measuring mat weights following filtration, and drying of the mycelium at 110°C. Cell free filtrates were obtained by filtration through 0.45 μ pore size Millipore filters. Enzymatic activity was detected by measurement of release of reducing sugars (DISCHE 1955).

RESULTS

The contribution of the marine microflora to the killing of *E. coli* in sea water was established by adding that bacterium to sterile sea water with concentrated native marine bacteria added. The rate of kill of *E. coli* in the water was found to be directly proportional to the concentration of marine bacteria present. When 10^5/ml marine bacteria were added to the sea water the *E. coli* population was reduced from 10^6/ml to 10^2/ml in 4 days. Addition of only 10 marine bacteria/ml resulted in little or no kill of *E. coli* in the same time period.

The effect of marine location on the killing of *E. coli* was tested by comparing the addition of *E. coli* to unpolluted deep sea water and water which had been repeatedly exposed to *E. coli* to simulate a polluted condition. There was a 48 hour lag period before the *E. coli* inoculum began to die in the unpolluted water. On the other hand, the water repeatedly exposed to *E. coli* had an immediate capacity to kill *E. coli*, and no lag period was observed.

In a parallel study, the survival of *E. coli* was studied in natural sea water, in plankton sampled from the Atlantic Ocean 150 miles off the coast of Massachusetts, and in water from the Eel Pond at Woods Hole, Massachusetts. Bacterial counts were made from all samples. The data obtained are shown in Table 1. A direct relationship between the size of the bacterial population and the rate of kill of *E. coli* was observed. In the deep sea the bacterial population was low and the *E. coli* population declined from 10^9/ml to 10^7/ml in 5 days. However, the plankton in the deep sea, which had a large bacterial population associated with it, caused a much more rapid kill of *E. coli*. Water sampled from the Eel Pond at Woods Hole had a bacterial population of 10^5/ml and was very active in killing *E. coli*.

Two groups of microorganisms were found to develop during the decline of *E. coli* in the sea. An increase in numbers of bacteria, mostly pseudomonads, capable of enzymatically lysing *E. coli* cell walls was detected soon after *E. coli* began to decline in the water. A population of halophilic filterable parasitic bacteria similar to *Bdellovibrio bacteriovorus* (STOLP & STARR 1963), and capable of causing lysis of *E. coli* also developed in response to the addition of *E. coli* to sea water.

The kill of non-marine fungi in the sea appears to be similarly linked to the activities of the marine microflora. Data was obtained which showed a direct relationship between the size of the marine microflora and the rate of disintegration of *Pythium* mycelium. Sea water repeatedly exposed to *Pythium* increased its activity in killing that fungus. This highly active sea water was filtered and the filtrates were tested against whole fungus mycelium for lytic activity. It was demonstrated that polysaccharidases capable of lysing the fungal cell walls were excreted into the sea by marine bacteria. Anti-fungal enzyme activity increased following the addition of the fungus to the sea water.

DISCUSSION

The fate of *E. coli* carried into the sea in sewage appears to be related primarily to the size of the marine microflora at the point of discharge, and also to the history of previous pollution of that area with sewage.

Discharge of sewage into water where the marine microbial population is high and there is a previous history of pollution resulted in a rapid kill of the coliforms, and presumably of pathogenic bacteria carried in the sewage. Our data suggest that where sewage is discharged into clean unpolluted water, there is a lag period before a decline occurs, unless the sewage is carried away rapidly by ocean currents.

Non-marine fungi carried into the sea in run-off waters appear to follow the same rule. The kill is rapid in areas of the sea where there is a continuous flow of non-marine fungi into the sea because of the presence of an antagonistic microflora. When the flow is intermittent there is a lag period to allow for an increase in numbers of microorganisms which kill these fungi in the sea.

Two groups of microorganisms associated with the kill of *E. coli* in the sea have been detected. Bacteria which produce extracellular enzymes active against the *E. coli* cell walls are involved in the kill. A second and extremely interesting group of bacteria were found to be stimulated by addition of *E. coli* to sea water. These are halophilic forms of a filterable parasitic bacterium similar to *Bdellovibrio bacteriovorus* isolated from soil (STOLP & STARR 1963). The marine bacteria cause lysis of *E. coli*. However, it is not yet clear if the bacteria producing extracellular enzymes or the parasitic bacteria are ecologically more important in the killing of *E. coli* in the sea, or if other as yet undetected groups of microorganisms are involved.

The kill of *Pythium* in sea water appears to be caused by a group of bacteria which develop in response to the addition of that fungus. These bacteria act against the fungus by producing enzymes which lyse the hyphal cell wall. The data suggest that these are the only microorganisms responsible for the disintegration of *Pythium* in the sea.

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Table 1. Die-off of E. *coli* in sea water from different locations. The deap-sea and plankton samples were taken from the Atlantic Ocean at a distance of 150 miles off Massachusetts.

Sample	Total count of Bacteria	*E. coli* count in sea water	
		Initial	5 days
Woods Hole	10^5/ml	10^9/ml	10^4/ml
Deep sea surface	10^3/ml	10^9/ml	10^7/ml
Deep sea 50 metre depth	10^3/ml	10^9/ml	10^7/ml
Plankton	10^6/ml	10^9/ml	10^4/ml

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FULL PAPER TO APPEAR IN TETRAHEDRON LETTERS 1968

BACTERIAL LYSIS OF NON-MARINE MICROORGANISMS IN SEA WATER

C. Wirsen and R. Mitchell

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Division of Engineering and Applied Physics
Harvard University
Cambridge, Massachusetts

There is a continuous pollution of the sea by pathogenic microorganisms carried in sewage and runoff water. Yet we rarely hear of cases of disease caused by animal pathogens carried into the ocean, because of the rapid disappearance of such organisms. A number of mechanisms have been proposed to account for this effect (Jones, 1963; Pramer et al, 1963; Vaccaro et al, 1950).

We have been concerned in our laboratory with the contribution of the native marine microflora to the killing of intestinal bacteria, represented by Escherichia coli, and of soil fungi, represented by Pythium debaryanum, in sea water. Marine bacteria capable of enzymatically lysing E. coli in artificial sea water have been isolated (Mitchell and Nevo, 1965).

In the current investigation we first studied the relationship between the size of the marine microbial population and the rate of E. coli die-off. Large quantities of marine microorganisms were collected by centrifugation of fresh sea water. These microorganisms were added at different concentrations to autoclaved sea water in 250 ml Erlenmeyer flasks. E. coli was inoculated and the flasks were incubated at 25°C on a rotary shaker. Counts of E. coli were made at regular intervals. The results obtained show that there is a direct relationship between the size of the marine microflora and the rate and extent of die-off of E. coli in sea water.

The development of a specific antagonistic microbial population following inoculation of E. coli into sea water was indicated in experiments in which E. coli was re-inoculated into natural sea water 5 days after the initial

inoculum. The results show that the lag period in the die-off of E. coli was virtually eliminated and the slope of the die-off curve was significantly sharper than that observed with the first inoculum.

In a parallel study, the survival of E. coli was investigated in sea water samples taken from the Atlantic Ocean 150 miles off Cape Cod, Mass. E. coli populations declined from 10^9/ml to 10^7/ml in 5 days in those samples in which the total bacterial population was approximately 10^3 bacteria/ml, as determined by plate counts. By comparison, the E. coli population declined from 10^9/ml to 10^4/ml in 5 days in sea water sampled from a harbor at Woods Hole, Mass. The harbor population was approximately 10^6/ml. Thus it appears that the rate of kill of E. coli under natural conditions in the sea is at least partially controlled by the size of the native marine microbial population.

Phase contrast microscopy revealed the presence of large numbers of vibrios in filtrates following exposure to E. coli. Very few filterable vibrios were present in natural sea water before exposure to E. coli. The vibrios varied in size from approximately 0.2μ to 0.5μ long and were highly motile. They yielded plaques in 3 days when sea water containing them was added to double layer plates containing E. coli and autoclaved sea water in 0.7% agar and incubated at 25°C. These vibrios strongly resembled the parasitic filterable bacterium, Bdellovibrio bacteriovorus (Stolp and Starr, 1963).

Our evidence suggests that a microbial population capable of lysing E. coli is present in the sea. It appears that a large population of these parasitic bacteria develops in sea water contaminated with E. coli. Whether these bacteria alone are responsible for the killing of E. coli and other gram negative bacteria, or if they act together with other microorganisms in

the sea remains to be determined.

Previous studies have shown that non-indigenous fungi in soil are enzymatically degraded by the native microflora (Mitchell and Alexander, 1963; Skujins et al 1965).

We initially added mycelial mats of Pythium to natural sea water and also to sea water containing a tenfold concentration of marine microorganisms. Figure 1 shows that in static culture the fungus was slowly degraded in natural sea water and more rapidly in sea water containing a higher concentration of marine microorganisms. No degradation occurred in autoclaved sea water.

FIG. I

The Effect of Natural and Concentrated Sea Water on Pythium in Static Culture.

These data indicate a direct contribution by the marine microflora to the disintegration of the fungal mycelia. Typical photographs of Pythium mycelium shaken in sterile and non-sterile sea water are shown in Figure 2. Degradation

of the mycelium in sterile sea water for 5 weeks had no obvious adverse effect on the fungus while in natural sea water the fungal mat was degraded in 10 days.

FIG. 2

Left. Pythium Mat Shaken in Autoclaved Sea Water for 5 Weeks.

Right. Pythium Mat Shaken in Natural Sea Water for 10 Days.

It should be noted that the degradation time was cut in half when the mats were shaken.

Thus, it appears that there is also a native marine microflora capable of degrading this fungus. Evidence for this hypothesis was strengthened by results obtained when a second inoculum of Pythium was placed in the Erlenmeyer flask following disintegration of the first mycelial mat. The second inoculum was degraded in 6 days. These data indicate that the first inoculum of Pythium stimulated the development of an antagonistic microflora which rapidly disintegrated the second inoculum of the fungus.

In subsequent experiments the ability of cell-free filtrates of sea water to enzymatically degrade whole Pythium mycelium was tested. We used sea water

which had been exposed to and had degraded two consecutive inocula of Pythium so as to obtain maximal activity. When the second inoculum was completely degraded the sea water was filtered through 0.45μ pore size Millipore filters. The filtered sea water, which was found to be sterile, was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 were retained by the ultrafiltration membrane. Concentration was achieved within approximately one hour in a single step without the need for dialysis, using this method.

These concentrated cell-free filtrates were tested for enzymatic activity against whole washed Pythium mycelium. Pythium cell walls contain a high percentage of glucans so activity was measured by assaying for release of reducing sugars. The results obtained showed that reducing sugars were released from the mycelium within one hour after the addition of filtered sea water. This enzymatic activity was substantially increased following tenfold concentration of the materials having a molecular weight higher than 10,000. The enzymes were inactivated by heating to 100°C for 20 minutes.

These data point to the importance of enzymes produced by marine microorganisms in suppressing non-marine microorganisms in the sea. In a subsequent study, pure cultures of bacteria capable of degrading Pythium were isolated from the sea. These bacteria yielded results similar to those observed in natural sea water, However, the degradation in pure culture was more rapid. The bacteria apparently caused lysis of the mycelial form of the fungus by enzymatically degrading the cell walls of the fungus. The enzymes involved in this lytic process are still unknown. However, it seems reasonable to suppose that more than one enzyme is involved.

In summary, we have presented evidence indicating that Escherichia coli and Pythium debaryanum are lysed by the native microflora in sea water.

E. coli was lysed both by a group of bacteria which enzymatically lyse its cell walls and by a parasitic bacterium similar to Bdellovibrio. Lysis of the fungus was caused by a group of microorganisms which enzymatically degrade the cell walls.

In conclusion we would like to point out that lysis by the native marine microflora is only one parameter of the many possible processes which might account for the disappearance of non-marine microorganisms in the sea. Investigation of other as yet unknown processes offers a fascinating possibility for the future.

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There is a continuous pollution of the sea by pathogenic microorganisms carried in sewage and runoff water. Yet we rarely hear of cases of disease caused by animal pathogens carried into the ocean, because of the rapid disappearance of such organisms. A number of mechanisms have been proposed to account for this effect (Jones, 1963; Pramer et al, 1963; Vaccaro et al, 1950).

We have been concerned in our laboratory with the contribution of the native marine microflora to the killing of intestinal bacteria, represented by Escherichia coli, and of soil fungi, represented by Pythium debaryanum, in sea water. Marine bacteria capable of enzymatically lysing E. coli in artificial sea water have been isolated (Mitchell and Nevo, 1965).

In the current investigation we first studied the relationship between the size of the marine microbial population and the rate of E. coli die-off. Large quantities of marine microorganisms were collected by centrifugation of fresh sea water. These microorganisms were added at different concentrations to autoclaved sea water in 250 ml Erlenmeyer flasks. E. coli was inoculated and the flasks were incubated at 25°C on a rotary shaker. Counts of E. coli were made at regular intervals. The results obtained show that there is a direct relationship between the size of the marine microflora and the rate and extent of die-off of E. coli in sea water.

The development of a specific antagonistic microbial population following inoculation of E. coli into sea water was indicated in experiments in which E. coli was re-inoculated into natural sea water 5 days after the initial

inoculum. The results show that the lag period in the die-off of E. coli was virtually eliminated and the slope of the die-off curve was significantly sharper than that observed with the first inoculum.

In a parallel study, the survival of E. coli was investigated in sea water samples taken from the Atlantic Ocean 150 miles off Cape Cod, Mass. E. coli populations declined from 10^9/ml to 10^7/ml in 5 days in those samples in which the total bacterial population was approximately 10^3 bacteria/ml, as determined by plate counts. By comparison, the E. coli population declined from 10^9/ml to 10^4/ml in 5 days in sea water sampled from a harbor at Woods Hole, Mass. The harbor population was approximately 10^6/ml. Thus it appears that the rate of kill of E. coli under natural conditions in the sea is at least partially controlled by the size of the native marine microbial population.

Phase contrast microscopy revealed the presence of large numbers of vibrios in filtrates following exposure to E. coli. Very few filterable vibrios were present in natural sea water before exposure to E. coli. The vibrios varied in size from approximately 0.2μ to 0.5μ long and were highly motile. They yielded plaques in 3 days when sea water containing them was added to double layer plates containing E. coli and autoclaved sea water to 0.7% agar and incubated at 25°C. These vibrios strongly resembled the parasitic filterable bacterium, Bdellovibrio bacteriovorus (Stolp and Starr, 1963).

Our evidence suggests that a microbial population capable of lysing E. coli is present in the sea. It appears that a large population of these parasitic bacteria develops in sea water contaminated with E. coli. Whether these bacteria alone are responsible for the killing of E. coli and other gram negative bacteria, or if they act together with other microorganisms in

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the sea remains to be determined.

Previous studies have shown that non-indigenous fungi in soil are enzymatically degraded by the native microflora (Mitchell and Alexander, 1963; Skujins et al 1965).

We initially added mycelial mats of Pythium to natural sea water and also to sea water containing a tenfold concentration of marine microorganisms. Figure 1 shows that in static culture the fungus was slowly degraded in natural sea water and more rapidly in sea water containing a higher concentration of marine microorganisms. No degradation occurred in autoclaved sea water.

FIG. I

The Effect of Natural and Concentrated Sea Water on Pythium in Static Culture.

These data indicate a direct contribution by the marine microflora to the disintegration of the fungal mycelia. Typical photographs of Pythium mycelium shaken in sterile and non-sterile sea water are shown in Figure 2. Deserica

of the mycelium in sterile sea water for 5 weeks had no obvious adverse effect on the fungus while in natural sea water the fungal mat was degraded in 10 days.

FIG. 2

Left. Pythium Mat Shaken in Autoclaved Sea Water for 5 Weeks.

Right. Pythium Mat Shaken in Natural Sea Water for 10 Days.

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It should be noted that the degradation time was cut in half when the mats were shaken.

Thus, it appears that there is also a native marine microflora capable of degrading this fungus. Evidence for this hypothesis was strengthened by results obtained when a second inoculum of Pythium was placed in the Erlenmeyer flask following disintegration of the first mycelial mat. The second inoculum was degraded in 6 days. These data indicate that the first inoculum of Pythium stimulated the development of an antagonistic microflora which rapidly disintegrated the second inoculum of the fungus.

In subsequent experiments the ability of cell-free filtrates of sea water to enzymatically degrade whole Pythium mycelium was tested. We used sea water

which had been exposed to and had degraded two consecutive inocula of Pythium so as to obtain maximal activity. When the second inoculum was completely degraded the sea water was filtered through 0.45μ pore size Millipore filters. The filtered sea water, which was found to be sterile, was concentrated by reducing the volume in a reverse osmosis ultrafiltration cell. Molecules with a molecular weight greater than 10,000 were retained by the ultrafiltration membrane. Concentration was achieved within approximately one hour in a single step without the need for dialysis, using this method.

These concentrated cell-free filtrates were tested for enzymatic activity against whole washed Pythium mycelium. Pythium cell walls contain a high percentage of glucans so activity was measured by assaying for release of reducing sugars. The results obtained showed that reducing sugars were released from the mycelium within one hour after the addition of filtered sea water. This enzymatic activity was substantially increased following tenfold concentration of the materials having a molecular weight higher than 10,000. The enzymes were inactivated by heating to 100°C for 20 minutes.

These data point to the importance of enzymes produced by marine microorganisms in suppressing non-marine microorganisms in the sea. In a subsequent study, pure cultures of bacteria capable of degrading Pythium were isolated from the sea. These bacteria yielded results similar to those obtained in natural sea water, However, the degradation in pure culture was more rapid. The bacteria apparently caused lysis of the mycelial form of the fungus by enzymatically degrading the cell walls of the fungus. The enzymes involved in this lytic process are still unknown. However, it seems reasonable to suppose that more than one enzyme is involved.

In summary, we have presented evidence indicating that Escherichia coli and Pythium debaryanum are lysed by the native microflora in sea water.

E. coli was lysed both by a group of bacteria which enzymatically lyse the cell walls and by a parasitic bacterium similar to Bdellovibrio. Lysis of the fungus was caused by a group of microorganisms which enzymatically degrade the cell walls.

In conclusion we would like to point out that lysis by the native marine microflora is only one parameter of the many possible processes which might account for the disappearance of non-marine microorganisms in the sea. Investigation of other as yet unknown processes offers a fascinating possibility for the future.

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